

## REMARKS

Claims 12, 14-23, 26, and 29-32 remain in the application for further prosecution.

Claims 24, 25, 27 and 28 have been cancelled. Claims 33-36 have been added. They relate to Claims 15-17 and 29-32 and are supported by Example 7 and 8.

### **Election/Restrictions**

The Examiner has made final the restriction requirement on the basis that Tretz et al teach monoclonal antibodies raised from purified UTI. However, Tretz et al used HI-30, which is the same or closely related to “bikunin”, having a molecular weight of about 33 kDa. The Applicant’s monoclonal antibodies were raised from purified “uristatin”, which is a recently identified UTI having a molecular weight of about 17 kDa. Thus the monoclonal antibodies of the application are novel and useful in detecting uristatin, and in detecting fractions of uristatin, which have been designated as uristation-1 and uristation-2. Thus, reconsideration is requested since Groups I and II relate to a single general inventive concept, contrary to the Examiner’s position.

### **Specification**

Corrections have been made to the specification as requested by the Examiner. In particular, where trademarks have been registered the symbol ® has been added.

In the published application it appears that the amendments to the specification were not included, while the claims do reflect the preliminary amendment of April 6, 2006. It is important that the specification is corrected where necessary. The Applicants ask for confirmation that the specification has been amended in the Examiner’s file. The specification was amended under PCT Article 34 and Rule 66 in a paper dated August 12, 2005 and further amended in the preliminary amendment of April 6, 2006. The paragraphs replaced in the above amendments to

the Specification were not amended previously, except for the paragraph on page 11, lines 5-14 which contains a minor clarifying amendment made under Article 34 and Rule 66 of PCT.

### **Rejections under 35 U.S.C. 112, Second Paragraph**

6.A The Examiner objected to the terms “strongly detected” and “binds strongly” as rendering Claims 15-17 indefinite. These claims have been amended to remove those terms and to substitute “preferentially bound” or “preferentially binds”. It should be understood that whether one refers to strongly detected or the more precise term “preferentially bound” the actual measurement of the relative amounts of UTI’s will depend on the means used to test the monoclonal antibodies. It is the degree of binding to UTI’s, without cross-reactivity to P- $\alpha$ -I and I- $\alpha$ -I pro-inhibitors, that determines the usefulness of the monoclonal antibodies. Thus, whether Western blots, ELISA or SELDI tests are employed, one obtains results which measure the relative amounts of UTI’s present in a biological sample in order to relate those results to the presence of disease.

6.B Claim 19 has been rejected as indefinite as being an omnibus claim. Applicant’s disagree that Claim 19 is an omnibus claim. It contains no uncertainty as to its coverage. All that was intended was to simplify Claim 19 by using acronyms for known immunoassays, as defined in the specification at page 10. This has been clarified in the present amendments.

6.C Claims 12, 14-23 and 25 have been rejected as being incomplete, in lacking essential steps. Claim 12 has been amended above by adding a step relating to the assaying of a biological fluid for UTI’s preferentially bound to monoclonal antibodies raised from purified uristatin.

6.D Claim 25 was rejected as lacking antecedent basis for the limitation “one of said hybridoma”. This rejection has been obviated by canceling claim 25.

8. Claims 24 and 27 have been rejected for failing to comply with the written description requirement. The Examiner argues “the specification does not appear to provide an adequate written description for all UTIs of the claimed monoclonal antibodies” (emphasis supplied). Claims 24 and 27 have been canceled, thus obviating this rejection.

The Applicants note that the problem being addressed was how one could detect or measure the presence of specific UTIs rather than all of them, which was done by prior art methods discussed at page 2 and by polyclonal antibodies discussed at page 3. Previous reports of the use of monoclonal antibodies were discussed at pages 3 and 4. By raising monoclonal antibodies from hybridomas derived from purified uristatin, a recently identified UTI, the Applicants made it possible to identify certain lower molecular weight UTI’s, but avoiding cross-reactivity with the pro-inhibitors.

The Lederman and Li articles cited by the Examiner are of interest, but not directly pertinent to the present invention. They only suggest that analogs may differ in their response to monoclonal antibodies or other biological activity.

9. Claims 24 and 27 also have been rejected as not being enabled for monoclonal antibodies having specificity for all UTIs, as defined in the specification. The Examiner argues that the specification was enabled for three monoclonal antibodies shown to preferentially bind uristatins, Bikunin, AMBK, and THP. The other UTIs, as defined were considered to be unpredictable and would require an undue amount of experimentation to evaluate the capability of the genus of antibodies for all species of UTIs. Both claims have been canceled, obviating the rejection.

The present invention is novel in that monoclonal antibodies were raised from purified uristatin, which is a UTI that has only recently been identified. By using the new antibodies the

binding of lower molecular weight UTIs, but not the pro-inhibitors P- $\alpha$ -I and I- $\alpha$ -I, as has been demonstrated in Example 6. As stated at page 5, line 32 to page 6, line 3, "These monoclonal antibodies are characterized by their ability to allow direct or indirect measurement of urinary trypsin inhibitors ... in the presence of other common proteins in urine or serum ... and non-inhibitory urinary trypsin inhibitors ... such as I- $\alpha$ -I and P- $\alpha$ -I." See also page 13, lines 22 – 23, "an immunoassay which detects only specific forms of UTI's, such as Uristatin, Bikunin, and AMBK, but not P- $\alpha$ -I, I- $\alpha$ -I, or THP would have clinical utility."

Claims 12 and 13-32 (sic, 14-32?) have been rejected as not enabling the correlation of any disease with measured UTIs. This rejection should be reconsidered since the claims do not claim assaying of a biological fluid for a disease as characterized by measuring UTI's. Rather, the claims describe a method of assaying a biological fluid for UTI's using monoclonal antibodies raised from purified uristatin. As the application explains, UTI's have been related to inflammation or infection and may be associated with many diseases. However, the effectiveness of the present monoclonal antibodies compared with other prior art test methods recently has been shown in a 2006 paper authored by a group including several of the Applicants. (copy enclosed). More precise correlation of UTIs with specific diseases has yet to be determined. As there reported "New methods now allow the direct measurement of specific forms of uTi with monoclonal antibodies in urine and blood. This is the first report of monoclonal antibodies that do not show an interfering cross reactivity to prohibitors in blood and THP in urine". (See page 14, left column).

The last sentence states the novelty and usefulness of the method being claimed. Cross-reactivity with interfering proteins can be avoided, thereby making possible identification of the uristatins and bikunin in biological samples and potentially, correlation with specific diseases.

Claims 14-19, 22-23, and 26-32 have been rejected for failure to comply with the enablement requirement, in that the new hybridomas are not available to the public. The Applicants have submitted a copy of the deposit receipt from ATCC and a statement under 37 CFR 1.804(b) by one of the inventors. Those papers were received at the USPTO on March 12, 2007. The above amendments include description of the deposit as required by 37CFR 1.809(d), which should complete the requirements.

### **Rejection under 35 USC 102**

Claims 12, 20-21 and 25 have been rejected under 35 USC 102(b) as anticipated by Papuashvili et al (US 6,242,197) (“Papuashvili”). Reconsideration is requested. The ‘197 reference was distinguished in the Preliminary Amendment of April 6, 2006. The critical difference lies in the type of UTI used to raise antibodies. Papuashvili raised her monoclonal antibodies using an “acidic glycoprotein” having molecular weight of about 44 kDa (see column 3, lines 17-34 and column 5, lines 12-16) and not the purified uristatin of the Applicants. Thus the antibodies of the ‘197 patent are not inherently the same as those used in the claimed method. The monoclonal antibodies raised from purified uristatin (molecular weight about 17 kDa) are unique in that uristatin was only recently recognized as the fragment of bikunin and the monoclonal antibodies have more specificity, since they are not cross-reactive with higher molecular weight proteins that interfere with assays of UTIs.

It should be clear from the Papuashvili patent that UTI was defined as being “an acidic glyco protein” having a molecular weight of about 44kDa. Thus, when the monoclonal antibodies were raised from a material considered to be a UTI, as it was defined, it was assumed that the monoclonal antibodies were binding the same UTI, which was related to the AID’s virus. There was no suggestion that individual fractions of urinary trypsin inhibitors were present or

distinguished from each other. This is evident from the fact that Papuashvili fails to distinguish between monoclonal antibodies and polyclonal antibodies. Clearly, all forms of UTI were being detected.

The Applicants teach that UTI includes many forms, use a purified fraction containing substantially uristatin to raise monoclonal antibodies, and demonstrate that their monoclonal antibodies bind to several low molecular weight UTIs. Therefore the Applicant's method is not anticipated. Furthermore, it could not be considered obvious from the limited disclosure of the '197 patent. In view of the amendments and the discussion above, the claims are believed to be in condition for allowance. If the Examiner believes that further consideration is needed, she is invited to contact the Applicant's attorney to arrange a telephone interview.

Respectfully submitted,

7/23/07  
Date



Harold N. Wells  
Reg. No. 26,044  
Kelley Drye & Warren LLP  
333 West Wacker Drive, Suite 2600  
Chicago, IL 60606  
Attorney for Applicants  
Tel.: (312) 857-2336

# Immunological evaluation of urinary trypsin inhibitors in blood and urine: Role of N- & O-linked glycoproteins

Michael J. Pugia · Saeed A. Jortani · Manju Basu ·  
Ronald Sommer · Hai-Hang Kuo · Solomon Murphy ·  
Doug Williamson · James Vranish · Patrick J. Boyle ·  
Danny Budzinski · Roland Valdes Jr. · Subhash C. Basu

Published online: 18 November 2006  
© Springer Science + Business Media, LLC 2006

**Abstract** Urinary trypsin inhibitors (uTi) suppress serine proteases during inflammation. After liberation from proinhibitors (P-alpha-I and I-alpha-I) by the white blood cell (WBC) response, uTi readily pass through the kidneys into urine. A key uTi, bikunin, is attached to O-linked and N-linked glycoconjugates. Recently, uTi inhibitors, called uristatins, were found to lack the O-linked glycoconjugates. Monoclonal antibodies were produced using purified uristatin and screened for binding differences to uristatin, bikunin, P- $\alpha$ -I, and I- $\alpha$ -I. Antibody-binding patterns were characterized using immunoaffinity binding onto protein-chip surfaces and analysis by Surface Enhanced Laser Desorption/Ionization mass spectrometry (SELDI), using specimens from patients and from purified uTi standards. Antibodies were developed and used in an enzyme-linked immunosorbent assay (ELISA) method for uTi measurement in urine and plasma specimens. ELISA was performed on specimens from normal, presumed healthy, controls and from patients who had been screened for inflammation using a high sensitivity C-reactive protein (CRP) test and a complete blood count (CBC). Polyclonal antibody against

uTi showed cross-reactivity with the Tamm–Horsfall protein (THP) and with proinhibitors. Screening of anti-uTi monoclonal antibodies (Mab) revealed antibodies that did not cross-react with either of the above, thus providing a tool to measure both uristatin and bikunin in urine with Mab 3G5 and in plasma with Mab 5D11. The monoclonal antibody 5D11 cross-reacts with specific N-linked glycoconjugates of uristatin present in plasma. In ca 96% of healthy adults, uTi were present at <12 mg/l in urine and <4 mg/l in plasma. We also found that patients with an inflammation and a CRP of >2.0 mg/l had higher urinary concentrations of uTi than the control population in every subject. Free uristatin and bikunin pass readily into urine and are primarily bound to heavy chains that constitute the proinhibitor form in plasma.

**Keywords** Uristatin · Bikunin · Urinary trypsin inhibitors · N-linked glycoproteins · O-linked chondroitin sulfate ·  $\alpha$ 1-Acid glycoprotein · Tamm–Horsfall protein · Monoclonal antibody · ELISA · Immunoassay · C-reactive protein · SELDI

M. J. Pugia · R. Sommer · H.-H. Kuo · S. Murphy · D. Williamson  
Diagnostic Business Group, Bayer Healthcare LLC,  
Elkhart, IN, USA

S. A. Jortani · R. Valdes Jr.  
Department of Pathology and Laboratory Medicine,  
University of Louisville School of Medicine,  
Louisville, KY, USA

M. J. Pugia · M. Basu · J. Vranish · P. J. Boyle ·  
D. Budzinski · S. C. Basu (✉)  
Department of Chemistry and Biochemistry,  
University of Notre Dame,  
Notre Dame, IN 46556, USA  
e-mail: sbasu@nd.edu

## Abbreviations

AMBK	$\alpha$ -1-Microglobulin/Bikunin precursor protein
ACT	$\alpha$ -1-Antitrypsin
AGP	Alpha-1-acid glycoprotein (orsomucoid)
ALP	Alkaline phosphatase
AMG	$\alpha$ -1-Microglobulin
BAPNA	N- $\alpha$ -Benzoyl-DL-arginine-4-nitroanilide
CRP	C-Reactive protein
CBC	Complete blood cell count
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HAT	Hypoxanthine–aminophen–thyidine

HAS	Human serum albumin
IMDM	Iscove's modified Dulbecco's medium
Mab	Monoclonal antibody
Pab	Polyclonal antibody
P- $\alpha$ -I	Pre- $\alpha$ -inhibitor
I- $\alpha$ -I	Inter- $\alpha$ -inhibitor
PEG	Polyethylene glycol
SELDI	Surface-enhanced laser desorption/ionization mass spectrometry
THP	Tamm–Horsfall protein (uromodulin)
uTi	Urinary trypsin inhibitor
WBC	White blood cell count
ZP	Zona pellucida

## Introduction

The presence of increased uTi concentrations is a significant finding in infection, acute and chronic inflammation, neoplasia and kidney diseases [1]. Bikunin is the highest molecular weight form of uTis and consists of two Kunitz inhibitor domains and two glycoconjugate chains [1, 2]. Proteases cause the release of bikunin from the interleukin- $\alpha$ -inhibitor (I- $\alpha$ -I) and the preinterleukin- $\alpha$ -inhibitor (P- $\alpha$ -I) during inflammation [3]. Bikunin is also part of the precursor protein pre-alpha-1-microglobulin/bikunin (AMBK). New forms of urinary trypsin inhibitors (e.g., uristatin) were discovered recently in patients with an acute phase reaction [4–6]. Uristatins contain either of the Kunitz inhibitor domains; however, they always lack the O-linked glycoconjugate chain. These forms were significantly more prevalent in patients' urines with the above conditions than in healthy controls. Further, they inhibited the members of the trypsin family of serine proteases. Variations owing to fragmentation of uTi and the presence in the N-linked glycoconjugate were demonstrated by Western blot analysis with an uTi polyclonal antibody (Pab) [6].

All active urinary trypsin inhibitors in a urine specimen can be measured by inhibition of trypsin [4–6]. The diagnostic use of this measurement as a nonspecific test for infection or inflammation is similar to the finding with C-reactive protein (CRP), a complete blood count (CBC) or sedimentation rate [4–10]. The first dipstick method for detecting total urinary trypsin inhibitors is based on the inhibition of a newly found enzyme substrate [4–6]. This finding permits the application of the dipsticks to the identification of urinary tract infections and kidney diseases. The strip is a urine test alternative to a blood measurement for CRP. When uTi values are  $\geq 12.5$  mg/l, the test is predictive of upper respiratory infections and other infections owing to bacteria or viruses as well as of inflammatory disorders.

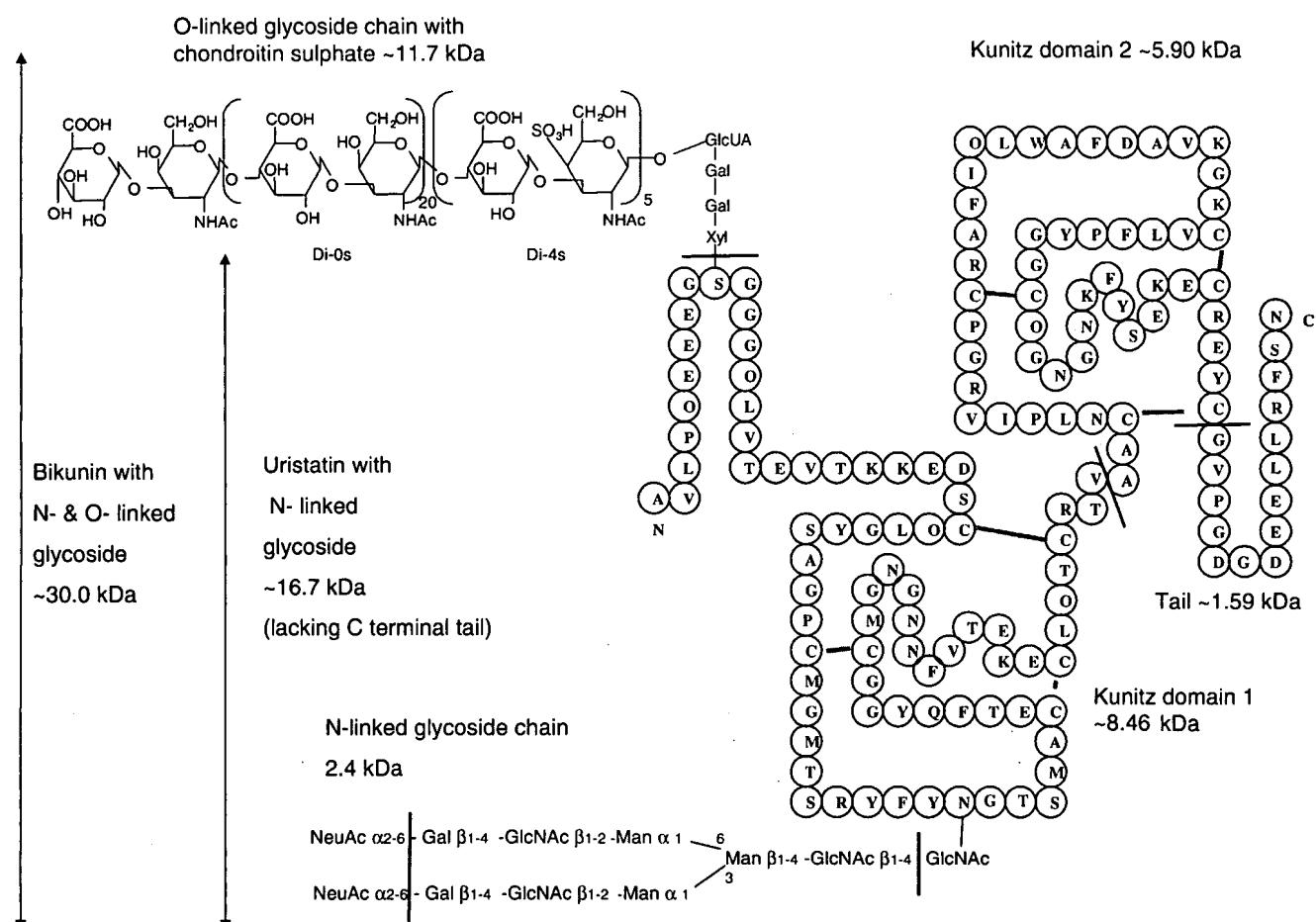
Immunoassays for uTi offer greater clinical sensitivity in identifying disease, especially inflammation. But to date, available immunoassays are unable to distinguish among the various forms of uTi from proinhibitors (I- $\alpha$ -I and P- $\alpha$ -I) [6, 11]. The cross-reactivity of polyclonal antibodies negates tests on blood specimens where I- $\alpha$ -I and P- $\alpha$ -I are present in both healthy and infected patients. In urine, polyclonal antibodies are more effective because P- $\alpha$ -I or I- $\alpha$ -I are not typically present but still cross-react with higher molecular weight forms found during a Western blot analysis [6]. Trefz *et al.* showed that a monoclonal antibody (IATIS) produced against I- $\alpha$ -I also recognized P- $\alpha$ -I, AMBK and Bikunin [11]. Report from Kobayashi *et al.* showed monoclonal antibodies raised against AMBK were reactive to uristatin, AMBK, and bikunin [12].

Our primary goal was to produce uTi monoclonal antibodies (Mab) that did not show the cross-reactivity of polyclonal antibodies (Pab). Three monoclonal (Mab) and one polyclonal (Pab) antibodies were selected and characterized for cross-reactivity by measuring protein binding (SELDI & ELISA techniques for analysis). Protein-chip array technology, also known as "surface-enhanced laser desorption/ionization time of flight mass spectroscopy (SELDI-TOF MS)", is useful for the study of complex protein mixtures and combines affinity chromatography with mass spectrometry. This permitted us to develop ELISA methods for the assay of uristatin and bikunin in either blood or urine. A second goal was to test for correlation of uTi with high-sensitivity CRP, CBC, and the inhibition assays for uTi in hospitalized patients with or without inflammation.

## Materials and methods

### Urinary trypsin inhibitor standards

Standards for uTi were obtained from patients with chronic renal failure [6] (SciPac, Sittingbourne, Kent, UK, product code P205-1) (Fig. 1). Many standards were characterized by SELDI analysis as described below, and were either comprised mostly of uristatin or were mixtures of uTi, including uristatin 1 and 2 (5.9 and 8.5 kDa), uristatin (17 kDa), bikunin (~30 kDa), traces of AMBK (66 kDa), THP (~80 kDa), P- $\alpha$ -I (~125 kDa), and I- $\alpha$ -I (~240 kDa). Lot 80\_117 contained 98% pure uristatin. Lot 124\_111 was 80% uristatin with 10% uristatin 1 and uristatin 2 and 10% of higher molecular weight bikunin molecules. Lot 20\_120 contained 20% uristatin, 50% bikunin, and 30% of high molecular weight bikunin. Standards in solutions stored for 19 weeks showed some changes at 4°C yielding larger amounts of the uristatin and smaller amounts of bikunin.



**Fig. 1** Structure of bikunin and uristatin

All lots were stable at  $-20^{\circ}\text{C}$  to  $-70^{\circ}\text{C}$  either in solution or as lyophilized solids.

#### Preparation and selection of monoclonal antibodies

BALB/c mice were immunized with 100  $\mu\text{g}$  of uristatin standard. After 1 month, ocular bleeds were taken, and titrated by ELISA against uristatin to assess the immune response. The mice showing the best response received a booster shot of 100  $\mu\text{g}$  uristatin/mouse. After 4 days, the mice were sacrificed and the spleenocytes were fused with SP2-0 Ag14 myeloma cells using PEG solution with a ratio of spleenocytes to myeloma cells of 5:1 and plated into 96-well plates using 50% PEG/HAT growth media. After 7 to 10 days of incubation at  $37^{\circ}\text{C}$ , fusion cultures were monitored for growth by feeding every 3 to 4 days with the HAT selection method followed by subculturing in HAT growth media. After 2 to 3 weeks, hybridoma colonies were tested for specific antibody production using ELISA for detection of an immune response to the uristatin. The colonies giving a positive response were transferred to 24-well plates for further expansion. The colonies testing

positive were further expanded in 6-well plates in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS) [13]. After expansion, the colonies were frozen at  $-70^{\circ}\text{C}$  and then transferred to liquid nitrogen for long-term storage.

Over 50 active monoclonal antibodies from three fusions were screened after expansion; they were grouped into three groups (A, B and C) by the similarity of their binding patterns of uTi standards. The best representative antibodies from each group were selected. For the selected mother colonies, cell culture supernatants were cloned out twice using the limiting dilution method to assure that the antibodies were monoclonal. The hybridomas secreting these monoclonal antibodies in Iscove's Modified Dulbecco's Medium (IMDM) containing 30% Fetal Bovine Serum (FBS) and 10% dimethyl Sulfoxide (DMSO) were deposited at the American Type Culture Collection and designated as hybridoma ATCC 421-3G5.4C5.3B6 (Mab A), ATCC 420-5G8.1A8.5C1 (Mab B) and ATCC 420-5D11.5G8.1E4 (Mab C). Alkaline phosphatase (ALK)-labeled conjugates of monoclonal antibody were prepared for use in ELISA assays.

## Characterization of monoclonal antibodies with SELDI

Monoclonal antibodies (Mab A–C) and previously described rabbit polyclonal antibodies (Pab D) [6] were tested with uTi standards and patients' urines on chip surfaces. The binding was estimated by Surface-Enhanced Laser Desorption/Ionization (SELDI) analysis on a SELDI PBS II time of flight mass spectrometer (Ciphergen, Fremont, California) to determine the mass to charge ratios ( $m/z$ ) for the proteins binding to the antibodies. Two urine specimens from patients were tested further: patient one was positive for a bacterial infection in blood and was toxemic; patient 2 was positive for a urinary tract infection by microbiological cultures [6]. Binding was measured on two types of surfaces (PS20 and RS100) using a standard incubation procedure [14]. The signal for each mass measurement was compared to the background noise to obtain the signal to noise ratios (S/N). Only masses with S/N ratios greater than 10 were accepted.

The SELDI procedure was as follows: Three microliter of 50 mmol/l NaHCO<sub>3</sub> (pH 8.0) was added to each spot on the protein chip and covered with a plate (i.e., a bioprocessor) to form sample wells followed by the addition of 1  $\mu$ l antibody (1 mg/ml) to each spot and incubated at room temperature for 2 h with shaking in a controlled-humidity chamber. The solution from each spot at that time was washed twice with 5  $\mu$ l of washing buffer (phosphate buffered saline (PBS) + 0.5% Triton detergent). The unbound sites were blocked with 5  $\mu$ l of either 2 mg/ml BSA (bovine serum albumin) or 1 mol/l ethanolamine. After incubation at room temperature the BSA or ethanolamine was discarded and the spots were washed twice with 5  $\mu$ l of washing buffer (PBS + 0.5% Triton). Five microliters of PBS was added to each spot and the chips were placed into the bioprocessor. An additional 10  $\mu$ l PBS as well as 10  $\mu$ l of the sample to be tested (or PBS as a control) were added to each well, followed by shaking the sealed wells at 4°C for 18 h. The wells were then washed with washing buffer and PBS and again shaken at room temperature for 2 min. The wells were rinsed twice with 300  $\mu$ l of deionized water saturated with sinapinic acid; this serves as an energy-absorbing molecule during protonation of proteins bound to the antibodies. The latter are attached to the surface of the chips. The chips containing the antibody-bound specimens were analyzed for binding mass using the SELDI mass spectrometer according to the manufacturer's instructions.

## Testing for cross-reactivity

The monoclonal antibodies A, B, and C as well as polyclonal antibody D raised against uTi were tested for cross-reactivity using ELISA against following glycopro-

teins: Tamm–Horsfall protein (THP), orsomucoid (Biomedical Technologies Stoughton, MA),  $\alpha$ -1-microglobulin (AMG; the Binding Site, Birmingham, UK),  $\alpha$ -1-antichymotrypsin (ACT; Research Diagnostics, Flanders NJ),  $\alpha$ -1-acid glycoprotein (AGP; orosomucoid, Sigma-Aldrich, St. Louis, MO), Human Serum Albumin (HSA; Sigma-Aldrich), and two glycosidic chains: chondroitin sulfate A and B (Sigma Aldrich).

Specific cross-reactants (5 ng/well) were first immobilized on microtiter plates (PN 3690 Corning Life Sciences, Acton, MA) overnight at 4°C. Plates were then treated with SuperBlock® (Pierce, Rockford IL) to reduce nonspecific bindings after which incubated with anti-uTi antibody (0.001 to 0.1  $\mu$ g/well) for another 30 min at 37°C. Alkaline phosphatase-conjugated goat anti-IgG conjugated (Sigma) was added to the wells and incubated for 30 min at 37°C. Each of the above binding steps are preceded by washing with Tris-buffered saline and 0.05% Tween-20 (TBS-Tween, Pierce). The antibody binding is visually estimated by reacting with *p*-nitrophenyl phosphate, the disodium salt (50  $\mu$ l PNPP 1-Step™ solution, Pierce), and the absorbance was read at 405 nm using a spectrophotometric micro titer-plate reader (SpectroMax, Molecular Devices Corporation, Sunnyvale, CA). Proteins with any binding to antibodies A to D were assayed again as mixtures with uTi standard at 0, 20, 40, 60, 80, and 100% to determine if binding was competitive. We estimated the cross-reactant's final time at the higher urine concentrations, up to 400 mg/dl, using the final competitive ELISA assay developed for the analysis of patients' specimens (See below).

## Enzyme-linked immunosorbent assays (ELISA) for clinical analysis

Patients' specimens were tested using a competitive or sandwich ELISA technique with calibrators for urine and plasma prepared in SuperBlock® blocking buffer at 0, 2.5, 5, 10, 20, 40, 60, 100 and 200 mg/l. The competitive format for Mab A–B and Pab D assay procedure is initiated by coating each well with 50  $\mu$ l of 0.1  $\mu$ g/ml uristatin (Lot 124-111) in TBS (Tris buffered saline). The plates were covered with adhesive tape, stored at 4°C overnight followed by washing five times with 160  $\mu$ l of TBS per well (Elx 405 Auto Plate Washer, Bioteck Instruments, Winooski, VT), blocked with 160  $\mu$ l of SuperBlocker® per well, incubated for 30 min at 25°C (JitterBug Microplate Shaker, Beokel Scientific, Feasterville, PA), and finally washed the plates five times with 160  $\mu$ l of TBS-Tween per well.

Calibrators and samples were diluted with antibody-alkaline phosphatase (ALP) conjugates in TBS using a Precision 2000 Automated Pipetting System (Bioteck Instruments). The stock conjugate concentration was 0.08  $\mu$ g/ml for Mab A and B and 0.40  $\mu$ g/ml for Pab D. Analysis of

urine required a 100-fold dilution of ALPMab<sub>(A&B)</sub> and 1,000-fold dilution for ALP-Pab<sub>(D)</sub>. A 1,000-fold dilution of the Mab was used for plasma assays. Diluted calibrator or specimen in 50 µl volumes were loaded into the coated wells, incubated at 25°C for two hours, and washed five times with 160 µl TBS-Tween per well. The sandwich format was used for Mab C by coating each well with 80 µl of 10 µg/ml of Mab C in TBS. Plates were covered with adhesive, stored at 4°C overnight, washed five times with 160 µl of TBS per well, blocked with 160 µl of SuperBlocker® per well, incubated for 30 min at 25°C, and washed five times with 160 µl of TBS-Tween per well. The calibrators and samples were diluted 1,000-fold with SuperBlocker for both urine and plasma assays. After dilution, 50 µl of calibrator or specimen were loaded into the coated wells, incubated at 25°C for 2 h, washed five times with 160 µl TBS-Tween per wells, and then 50 µl of 0.40 µg/ml ALP-Mab<sub>(D)</sub> (Pab D) conjugate was loaded into each well. The plate was incubated at 25°C for another 2 h followed by a second TBS-Tween wash cycle.

The antibody binding is estimated by incubation at 25°C for 30 m (JitterBug Shaker) after addition of PNPP (50 µl/well; 1-Step™ solution, Pierce), which is hydrolyzed by bound ALP conjugate, followed by addition of 25 µl 2 M NaOH to each well to stop the reaction. The plates were read at 405 nm on a FLx 800 microplate Reader (Biotek Instruments). The average absorbances and SD were calculated for the triplicate results. Unknowns were determined from standard curve after a sigmoidal nonlinear curve-fitting routine (variable slope) using the Prism 4 program (GraphPad Software). We analyzed each calibrator and specimen in triplicate using 96-well plates. Separate plates were used for each antibody method. Triplicates were spaced on the microplates as widely as possible for best assay precision. The CV was between 2.4 to 14.8% for all the calibration steps.

#### Specimen collection from patients and controls

We obtained urine and whole blood specimens from 188 hospitalized patients. Clean-catch midstream urines were obtained from all patients. The blood specimens were collected into EDTA-containing tubes (BD Vacutainer®) and immediately separated them into plasma and cells. Specimens were stored at 4°C until tested. If not tested within 4 h, storage was at -20°C. Inflammation was found in only 7 of 188 patients with a high sensitivity CRP result of ≥2.0 mg/l (BN II nephelometer Dade Behring, Deerfield IL). The control group of 181 patients without inflammation had an average CRP of 0.4±0.5 mg/l and an average white blood cell (WBC) count of 6,800±2,400 cell/µl (Cell-Dyne 4000, Abbott Diagnostics, Santa Clara, CA). There was only one result above 12,400 cell/µl, the upper

limit of abnormal leukocyte counts. The affected group (n=7) with a CRP of ≥2.0 mg/l had only a slightly elevated WBC of 9,100±2,900 cell/µl.

#### Inhibition assays for uTi

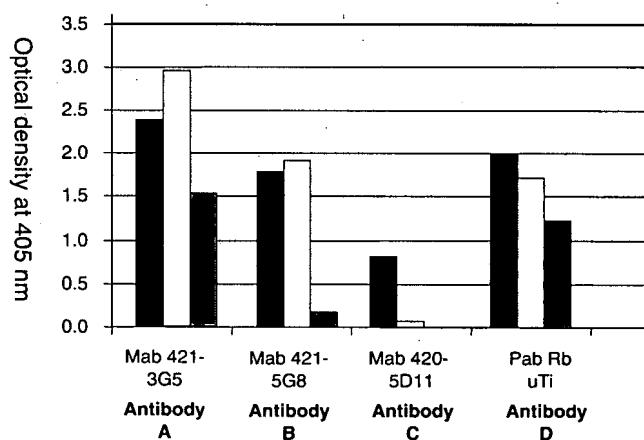
Inhibition assays using an arginine peptide substrate (BAPNA) and trypsin on a CX3 analyzer (Beckman) allowed the uTi to be determined in urine according to the previously published Cobas Fara II method [12]. Calibration using uTi standards at 0 (undetectable) and at <2.5, 6.25, 12.5, 25, 50, 75, and 100 mg/l permitted the assay calculation from a linear regression line. The precision of the Beckman analyzer (duplicates on five days) was 5.17±0.39 mg/l (SD) and 48.4±0.58 mg/l (SD). The nominal or "labeled" concentrations of the controls from the manufacturer were 6.25 mg/l and 50 mg/l.

We also performed a uTi dipstick test on the Clinitek® 50 strip reader (Bayer HealthCare) to obtain uristatin concentrations of 0 (undetectable at <6.25 mg/l), 12.5, 25, and 50 mg/l [6]. The strip measured uTi controls with values of 0 and 12.5 mg/l correctly 99% of the time for five determination/day over ten days.

## Results

#### Monoclonal antibody screening

Monoclonal antibodies were classified into three groups (A, B and C) based on their response to three lots of purified uTi standards (Fig. 2). Group A antibodies had the strongest



**Fig. 2** Antibody binding to urinary trypsin inhibitors standards. Shown are the typical binding patterns for monoclonal antibodies (A, B, C) and polyclonal antibody (D) to three lots of purified uTi standards; Lot 20\_120 (solid bars) contains 20% uristatin, 50% bikunin, and 30% of high molecular weights; Lot 124\_111 (open bars) was 80% uristatin with 10% uristatin-I and 2, and 10% of bikunin with higher molecular weight fragments present; and lot 80\_117 (gray bars) contained substantially only uristatin (98% pure)

binding affinity to uTi lot number 124\_111 over other lots. The reduced concentration of high molecular weight proteins in lot 124\_111 suggested these clones would be less reactive to P- $\alpha$ -I and I- $\alpha$ -I. Previously, Pab was found to be more reactive to these proinhibitory forms [6]. We selected clone 421-3G5 for further study as it had the strongest binding affinity of the group. Group B antibodies had equal binding affinity to lots 124\_111 and 20\_120. We chose clone 421\_5G8 as it had the lowest binding affinity to lot 80\_117. The group C antibody had a strong binding affinity for standards that contained a significant amount of proteins with molecular weights of 60 to 80 kDa. This is consistent with earlier findings with western blot using patients' clinical urines that showed Group C clone 420\_5D11 had bound strongly to protein in the 60 to 80 KDa range.

#### Surface-enhanced laser desorption/ionization (SELDI)

The SELDI results for the three Mab and the Pab are given in Table 1. The values represent the *m/z* (mass number) of proteins bound to the antibodies after reaction with the uTi standards and the urine of two patients with bacterial infections. This method determined the molecular weights of the bound forms and does not rely on any interpretation of the composition of the standard. The high sensitivity of

the SELDI method demonstrates antibody binding to many more forms than are detectable by comparison to standards. Those binding events of high affinity, frequency, or importance are indicated in italic bold numbers as determined by signal-to-noise ratios of binding events (Table 1). The secondary binding events are bold while weak binding events are in plain text.

As seen in Table 1, SELDI and uTi standards demonstrated that the primary binding for Mab A was strongest for the small uristatin 1 or 2 fragments, uristatin, and AMBK. All four antibodies bound to all of the above proteins, with the uristatins showing the strongest binding. We found no significant proinhibitor binding at the low concentrations found in the standards derived from urine. The primary binding for Mab B was with uristatin 1 or 2, less so for uristatin, no binding of bikunin, and weak proinhibitor binding. The primary binding for Mab C was strongest for THP, uristatin, and uristatin 1 or 2, and weaker for bikunin or AMBK. The polyclonal binding occurred with all forms including the proinhibitors and THP. All Mab exhibited strongest binding to uristatin 1 or 2 with molecular weights of 2.8, 3.5, and 3.9 kDa. The SELDI results also show several low affinity binding sites, but these were not strong enough to affect specificity.

The results in Table 1 using two patients' urines demonstrate the same primary binding patterns for anti-

**Table 1** Analysis of antibodies A–D using SELDI mass spectrometry after immunoaffinity binding to urinary trypsin inhibitors

Expected mass for antibody	Mass (kDa) of antigen bound to antibody <sup>a,b</sup>			
	Antibody A	Antibody B	Antibody C	Antibody D
Mab 421-3G5		Mab 421-5G8	Mab 420-5D11	Polyclonal Ab (anti-uristatin)
Uristatin 1 or 2. Average molecular weights of 5.9 and 8.5 kDa with kDa range 2–12	<b>2.82, 3.41, 3.48, 5.38, 5.57, 6.30, 7.24,</b> 3.5, 3.8, 4.0, 5.6, 6.0, 6.3, 8.0, <b>9.2, 9.8, 10.9</b>	<b>2.82, 3.41, 3.48,</b> 4.0, 5.4, 7.2, <b>9.18, 9.85</b>	<b>2.82, 3.0, 3.5, 4.7,</b> 3.7, 5.4, 5.6, 7.2, 3.7, 10.8	<b>2.8, 5.0, 5.4, 7.2, 10.7,</b> 3.5, 4.0, 5.9, 6.0, 6.3, 8.1, 10.7
Uristatin. 17 kDa, range 11–22	<b>11.8, 18.0,</b> 16.3, 13.4, 14.0	12.0, 14.0, 13.5, <b>21.1</b>	<b>11.9, 13.5, 21.1,</b> 23.0, 22.7	<b>13.4, 14.0,</b> 16.3 21.2, 22.6, 23.3, 33.4, 33.1, 35.2, 42.3
Bikunin. 30 kDa, range 21–46	<b>21.1,</b> 33.6, 35.2, 33.5, 45.9		23.3, 41.6	58.6
AMBK. 66 kDa, range 42–70	<b>67.0</b>	No significant peaks	No significant peaks	82.6, 79.2
THP. 85 kDa, 80–91 kDa	81.9, 91.1	No significant peaks	<b>80.2, 80.6, 81.9</b>	101.1, 103.7, 106.3, 120.0, 123.4, 133.0, 142.7
p-alpha-I. 125 kDa	No significant peaks	128.5, 132.1	No significant peaks	No significant peaks
I-alpha-I. 240 kDa	No significant peaks	No significant peaks	No significant peaks	No significant peaks

<sup>a</sup> Antibody binding observed with two patients' urine and the uTi standard. One patient was positive for a blood bacterial infection (sepsis) and the other was positive for a bacteriuria (urinary tract infection) as determined by microbiological cultures.

<sup>b</sup> Masses (kDa) rounded to nearest 0.1 kDa. Peaks with highest S/N relative to other peaks are reported in bold italic face, followed by bold face, and then plain text for lowest S/N binding events.

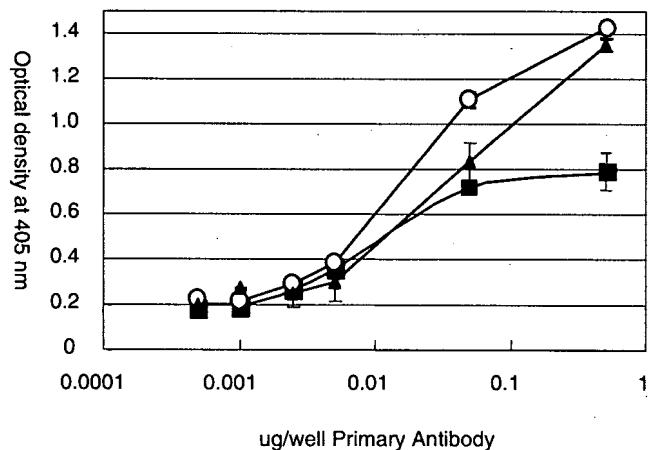
S/N Signal-to-noise ratio.

bodies A to D shown with the uTi standards. The SELDI results, however, also demonstrated several new forms of uristatin 1, uristatin 2, uristatin (containing both 1 & 2), and bikunin within the expected molecular weight ranges. In particular, primary binding to 2.8, 5.4, 7.2, 11.8, and 21.1 kDa proteins was observed in patients' specimens but not in the purified standards. Greater variations in the pathological conditions or the pooling and purification of standard to the target molecular weight could explain the difference. The SELDI binding also showed cross-reactivity to THP by the antibodies C and D in both the standards and patients' urines.

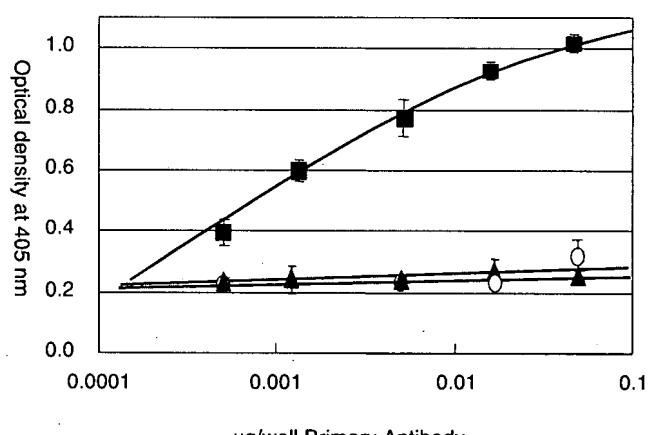
#### Cross-reactivity testing

Cross-reactivity testing of the three monoclonal antibodies as determined by ELISA using uTi, Tamm-Horsfall protein, and  $\alpha$ 1-acid glycoprotein is shown in Figs. 3, 4 and 5 and is in agreement with the findings by SELDI. All three Mabs bound preferentially to uTi with A and B showing better binding affinity than Mab C as seen in Fig. 3. No cross-reactivity of Mabs A or B was observed to either Tamm-Horsfall protein (THP) or  $\alpha$ -1-acid glycoprotein (AGP) while Mab C cross-reacted specifically to THP (Fig. 4) and AGP (Fig. 5). However the binding affinity of Mab C was ~30 times stronger for uTi than AGP. None of the monoclonal antibodies showed any specificity toward  $\alpha$ -1-microglobulin (AMG) of the precursor protein AMBK,  $\alpha$ -1-antichymotrypsin (ACT), human serum albumin (HSA), or chondroitin sulfate A and B. Pab were about 50% cross-reactive to THP and AGP (data not shown).

Competitive and ELISA methods when used for patients' specimens showed no cross reactivity to Mab A and B, with HSA (400 mg/dl), THP (4 g/l), AGM (400  $\mu$ g/ml), AGP (1 g/l) or ACT (400  $\mu$ g/ml). Mab C



**Fig. 3** Monoclonal antibodies A, B, and C reactivity to uristatin. Concentration of Mab A (circles), Mab B (triangles), and Mab C (squares) plotted versus the observed binding to uristatin (5 ng/well). Optical density at 405 nm reported after reaction of p-nitrophenyl phosphate (PNPP) with ALP

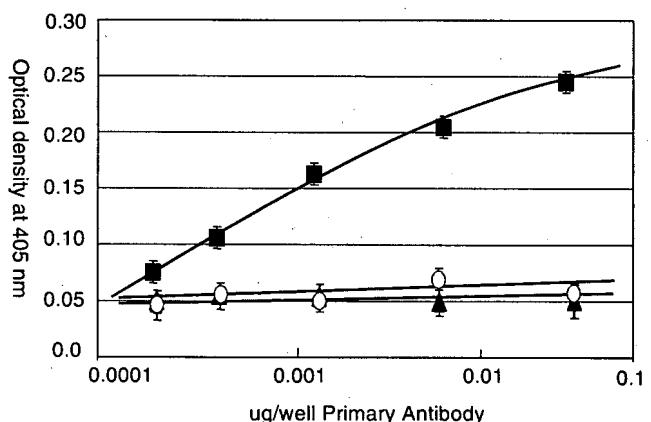


**Fig. 4** Monoclonal antibodies A, B, and C reactivity to Tamm-Horsfall protein. Concentration of Mab A (circles), Mab B (triangles) and Mab C (squares) plotted versus the binding observed to Tamm-Horsfall Protein (5 ng/well). Optical density at 405 nm reported after reaction of PNPP with ALP

remained cross reactive to THP (at 50 mg/l) and weakly cross reactive to AGP (at 2 g/l). Pab cross-reacted with THP at  $\geq$ 100 mg/l. This is the region of trace proteinuria.

#### Testing of normal and affected patients

Normal patients could be distinguished from those with inflammation with a CRP cutoff value of ( $< 2.0$  mg/l). Patients in the normal group ( $n=181$ ) had an average uTi by the quantitative inhibition assay of  $8.8 \pm 6.9$  mg/l while the population with inflammation ( $n=7$ ) had an average uTi of  $38.8 \pm 6.0$  mg/l. This agrees with previously reported cutoffs of 12 mg/l for those with inflammation [4–6]. The strip inhibition assay was negative for 153 of the normal group and positive for three of the affected group. The quantitative Beckman assay was negative for 150 of the normal group and positive for six of the affected group and



**Fig. 5** Monoclonal antibodies A, B, and C reactivity to alpha-1-glycoprotein. Concentration of Mab A (circles), Mab B (triangles) and Mab C (squares) plotted versus the binding observed to alpha-1-glycoprotein (50 ng/well). Optical density at 405 nm reported after the reaction of PMPP with ALP

value was ~80 mg/l). Blood values for uTi measured by Pab were also higher than the concentrations found in urine and no difference in the blood test results were found between patients and controls.

Blood uTi measurements could be made with Mab C and showed the free uTi to be 1 to 2% of the total proinhibitor concentration. We determined that a blood uTi test was not a predictor of general inflammation as measured by CRP. Using Mab C to assign reference values for blood free uTi (e.g., uristatin and bikunin), a cutoff of 4 mg/l was above 96% of the normal group. Comparison of blood and urine measurements supports the notion that free uTi passes readily through the glomeruli and that, in blood, these proinhibitor proteins are normally bound.

## Discussion

The SELDI analysis using uTi antibodies demonstrated that variations in uTi were far more common than previously reported [6]. Other groups have reported the insensitivity of SDS-PAGE analysis as compared to SELDI during studies of urine proteins in urolithiasis [14]. The variation of uTi was far greater in individual patient's specimens than expected with purified standards for whole uristatin and bikunin. The between-patient variation in uTi would be expected owing to the fragmentation of peptide structure by serine proteases released in patients with inflammation. Modification of the O-linked proteoglycan chain during inflammation has also been reported [15–17], and modifications of the complex N-linked glycan would be expected.

Uristatin fragments of uTi, previously identified, lacked the proteoglycan chain but were inhibitory toward serine proteases [6, 17–19]. These smaller uTi fragments of Kunitz domain either lacking or containing the N-linked glycan chain are commonly found in urine. Strong binding of the Mab A and B to these low molecular weight forms of uTi makes these ideal assays for urines where these small fragments are likely the best representation of the epitopes bound by each antibody. As these fragments are also common to I- $\alpha$ -1 and P- $\alpha$ -1, the assay of blood with Mab A or B measures these proinhibitors proteins as well as the smaller fragments. These proinhibitors do not readily pass into urine, and cross-reactivity is not a problem in urine testing, because the concentration for proinhibitors is lower [6].

The cross-reactivity of Mab C and Pab with THP and AGP is not due to common amino acid sequences since there is less than 10% homology [20]. THP has EGF-like light chains binding and ZP domains [21–25] while AGP has a lipocalin domain for binding certain steroid hormones, basic drug, IgG, heparin and vanilloid [26, 27]. Bikunin has two Kunitz domains for serine protease inhibition and no domains common to THP or AGP (Fig. 1). Bikunin,

uristatin, THP, and AGP all have a complex N-linked glycan in common that varies in the degree of fucosylation, sialylation, and branching [17–19].

The N-linked glycan of uTi has a biantennary structure (Fig. 1) [17–19]. The number of glycoforms of uTi is unknown. Tamm–Horsfall protein has five N-linked glycan chains that can be bi-, tri-, and tetra-antennary complexes [28–30]. The number of glycoforms of THP has also not been determined although it is known to be highly donor dependent. AGP has five N-linked glycan chains that can be bi-, tri-, and tetra-antennary complexes with 40 known glycoforms of which the biantennary complexes are rare [31–34].

The greater abundance of uTi forms now reported suggests a far greater possibility for a diagnostic role for fragments and their associated N-linked glycan chain in pathological conditions. Mab C appears to be ideal for measuring uTi fragments in blood through binding to the biantennary N-linked glycan of uTi. While this N-linked glycan is common in THP, the latter is only excreted in the proximal tubules of the kidney and is not found in serum [35]. In urine, cross-reactivity to THP is a concern. The weak cross reactivity to AGP does not appear to be a significant factor in blood. Even though AGP is present in substantial amounts in blood (ca 1 g/l), the biantennary species is an uncommon glycoform.

Glycoconjugates of the cross-reactive proteins (THP and AGP) play a role in the body's defense mechanisms. The N-glycan of THP has hemagglutination properties. The influenza virus binds to sialic acid through the neuraminidase-type glycoprotein on the virus cell membrane [35, 36]. THP also has one O-linked proteoglycan chain involved and is membrane-bound [28, 29]. One N-linked mannose chain has *E. coli* binding properties [37]. AGP is an acute-phase protein with membrane binding properties to modulate monocyte, granulocyte, and lymphocyte binding to the endothelial cells [26, 31]. The immunomodulatory activity of AGP is dependent on glycosylation with formation of sialyl Lewis X and leading to endothelial cell adhesion through P- and E-selectins thereby blocking immunocell adhesion [27]. The sialyl Lewis X is known to bind to the influenza virus [35]. Expression of sialyl Lewis X and bi-tetra-antennary complexes of AGP is known to increase early on with inflammation [26, 27] while greater fucosylation occurs with diabetes [34]. The role of the N-glycan of uTi is not known, although several acute phase processes could be implicated.

## Conclusion

New methods now allow the direct measurement of specific forms of uTi with monoclonal antibodies in urine and

agrees with the difference in detection limits previously reported [5, 6]. The quantitative inhibition assay is more accurate at the lower range and is more predictive of a CRP of 2.0 mg/l than the strips. The strip assay is more predictive of CRP values of  $\geq 10$  mg/ml [5].

Inhibition assays cannot be performed on blood owing to their nonspecific nature with any trypsin like enzyme or inhibitor in blood affecting the rate. Mab A and B and Pab were predictive of negative results by inhibition of urine assays. A positive Mab C was not predictive as expected by cross reactivity in urine to THP (false positives = 94). The negatives were as expected by the quantitative and strip assay. Inhibition assay positives showed better agreement with the immunoassay results for Mab A–B. The best correlation was with Mab A and the inhibition methods; here, specimens analyzed by the antibody assay were positive  $> 80\%$  of the time by the inhibition assays. We did not find false positives owing to exogenous inhibition in the immunoassay method.

Antibody tests were superior in separating those with and without inflammation as compared to the enzyme inhibition method (Table 2). Clinical assays of uTi in urine were best made with Mab A which detected all of the affected group and were negative in 167 of the normal group (Table 2). The normal population had an average uTi of  $3.6 \pm 5.4$  mg/l; the affected population had an average

uTi of  $28.0 \pm 7.0$  mg/l ( $p < 0.001$ ). Mab B and Pab were also predictive of six patients in the affected group and negative in 159 of the 164 of the normal group (Table 1). A cutoff of 7.8 mg/l gave the best separation of normal and affected patients. In practical use, Pab cannot be used because of the cross reactivity of THP causes many false positives. Proteinuria was absent from the patients and controls in this study (negative by protein dipstick). Mab C with 100% cross reactivity to THP was not useable for the urine tests. Only 81 of the control group were negative and just three of the seven in the affected group were detected.

The reference range for uTi in urine was similar for Mab A, B, and Pab. More than 96% of the normal group had a uTi value of less than 12 mg/l. The normal urinary range observed for Mab C was much higher with a 40 mg/l cutoff required to include 96% of the normal group. THP is normally present in urine with a reference range of up to about 50 mg/l.

Blood concentrations of uTi measured by Mab A or B were not significantly different between the normal and affected populations (Table 1). uTi values in whole blood were much higher than the concentrations found in urine suggesting cross-reactivity with proinhibitors. We confirmed this by western blot analysis. Using Mab A and B to assign a reference range for proinhibitors in blood, we found that 96% of the normals were 25 to 150 mg/l (average

**Table 2** Agreement of ELISA procedure (for antibodies A–D) and the high sensitivity CRP method, urine specimens

Urine specimens		Healthy controls (n = 181)			Patients with inflammation (n = 7)		
Primary proteins measured	Antibody method	Mean (mg/l)	SD (mg/l)	Controls correctly identified <sup>a,b</sup>	Mean (mg/l)	SD (mg/l)	Patients correctly identified <sup>b,c</sup>
uTi	Mab A	3.6	5.4	93%	24.9	34.9	100%
uTi	Mab B	4.1	6.1	88%	30.9	46.8	88%
THP	Mab C	12.6	13.1	NS	18.9	21.6	NS
uTi	Pab	3.6	5.6	91%	21.1	27.2	88%
Plasma specimens		Controls (n = 181)			Patients with inflammation (n = 7)		
Primary proteins measured	Antibody method	Mean (mg/l)	SD (mg/l)	Controls correctly identified <sup>b</sup>	Mean (mg/l)	SD (mg/l)	Controls correctly identified <sup>b</sup>
Proinhibitor	Mab A	76.4	37.6	NS	112.0	45.8	NS
Proinhibitor	Mab B	85.3	28.4	NS	99.7	13.7	NS
uTi	Mab C	1.0	1.1	NS	0.7	0.4	NS
Proinhibitor	Pab	26.4	16.9	NS	24.3	9.6	NS

<sup>a</sup>The percentage of patients (n = 181) with hsCRP  $< 2.0$  mg/l and urinary trypsin inhibitor  $< 7.8$  mg/l.

<sup>b</sup>NS ( $P > 0.001$ ) was observed between patients with and without hsCRP at 2.0 mg/l.

<sup>c</sup>The percentage of patients (n = 7) with hsCRP  $\geq 2.0$  mg/l and urinary trypsin inhibitor  $\geq 7.8$  mg/l.

NS no statistically significant difference.

blood. This is the first report of monoclonal antibodies that do not show an interfering cross reactivity to proinhibitors in blood and THP in urine. The antibodies have also been shown to serve as tools for uncovering the role of uTi and glycoconjugation with either ELISA or SELDI methods. To date it has not been shown whether certain glycoforms or uTi fragments occur more frequently in chronic inflammation, are more easily distinguished in acute inflammation, are more common in certain tissues, or are formed by certain pathogens. It is reasonable to assume that certain forms of uTi can result from chronic disease, and these new tools will help in identifying individuals with inflammation. This work should advance the biochemical understanding of inflammation.

**Acknowledgments** This study was supported in part by grants from Bayer Diagnostics to the University of Louisville and the University of Notre Dame. We would like to thank Ms. Loraine C. Souders for running the Beckman analyzer, CLINITEK 50, and for performing the ELISA clinical assays. We thank Mrs. Joan K. Clayborn, for the preparation of antibody-enzyme conjugates and Dr. Linda M. Anderson-Mauser for her support in antibody production. We also thank Mr. Edward P. Womack, MT (ASCP), and Dr. Christine Simmons for assistance with patient recruitment and SELDI analysis. Special thanks go to Rui Ma for organizing the Notre Dame group meetings. We dedicate this article to the memory of Dr. David J. Newman of S. W. Thames Institute for Renal Research, St. Helier Hospital, Surrey UK, who provided urine from renal patients for the uTi standards.

## References

1. Fries, E., Blom, A.M.: Bikunin—not just a plasma proteinase inhibitor. *Int. J. Biochem. Cell Biol.* **32**, 125–137 (2000)
2. Pugia, M.J., Lott, J.A.: Pathophysiology and diagnostic value of urinary trypsin inhibitors (review). *Clin. Chem. Lab. Med.* **43**, 1–16 (2005)
3. Fries, E., Kaczmarczyk, A.: Inter-alpha-inhibitor, hyaluronan and inflammation. *Acta Biochim. Pol.* **50**, 735–742 (2003)
4. Pugia, M.J., Takemura, T., Kuwajima, S., Suzuki, M., Cast, T.K., Profit, J.A., Schulman, L.S., Ohta, Y., Lott, J.A.: Clinical utility of a rapid test for uristatin. *Clin. Biochem.* **35**, 105–110 (2002)
5. Jortani, S.A., Pugia, M.J., Elin, R.J., Thomas, M., Womack, E.P., Cast, T., Valdes, R. Jr.: A sensitive non-invasive marker for diagnosis of infection and inflammation. *J. Clin. Lab. Anal.* **18**, 289–295 (2004)
6. Pugia, M.J., Sommer, R., Corey, P., et al.: The uristatin dipstick is useful in distinguishing upper respiratory from urinary tract infections. *Clin. Chim. Acta* **341**, 73–81 (2004)
7. Kuwajima, S., Izumi, Y., Noda, T., Kitao, H., Kishida, T., Naka, K., Okuda, K.: Automated measurement of urinary trypsin inhibitor, an acute phase reactant in urine. International Congress Series. *Prog. Clin. Biochem.* **991**, 317–320 (1992)
8. Kuwajima, S., Matsui, T., Kitahashi, S., Kishida, T., Noda, T., Izumi, Y., Naka, K., Okuda, K.: Urinary trypsin inhibitor and its clinical usefulness for diagnosis of acute phase reactant and renal disease. *Ensho* **9**, 175–182 (1989)
9. Takemura, T., Nakano, H., Kuwajima, S.: A clinical study of urinary trypsin inhibitor, an acute phase reactant in urine, in acute pediatric infectious diseases. *J. J. Inflam.* **14**, 53–57 (1994)
10. Mizon, C., Piva, F., Queyrel, V., Balduyck, M., Hachulla, E., Mizon, J.: Urinary bikunin determination provides insight into proteinase/proteinase inhibitor imbalance in patients with inflammatory diseases. *Clin. Chem. Lab. Med.* **40**, 579–586 (2002)
11. Trefz, G., Streit, B., Justus, C.W.E., Ebert, W., Kramer, M.D.: Establishment of an enzyme-linked immunosorbent assay for urinary trypsin inhibitor by using a monoclonal antibody. *J. Immunoassay* **12**, 347–369 (1991)
12. Kobayashi, H., Hirashima, Y., Sun, G.W., Fujie, M., Nishida, T., Takigawa, M., Terao, T.: Identity of urinary trypsin inhibitor-binding protein to link protein. *J. Biol. Chem.* **275**, 21185–21191 (2000)
13. Kohler, G., Milstein, C.: Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **6**, 511–519 (1976)
14. Cadieux, P.A., Keiko, D.T., Watterson, J.D., Burton, J.P., Howard, J.C., Knudsen, B.E., Gan, B.S., McCormick, J.K., Chambers, A. F., Denstedt, J.D., Reid, G.: Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS): a new proteomic urinary test for patients with urolithiasis. *J. Clin. Lab. Anal.* **18**, 170–175 (2004)
15. Mizon, J., Capon, C., Mizon, C., Lemoine, J., Rodié-Talbère, P.: In acute inflammation, the chondroitin-4 sulphate carried by bikunin is not only longer; it is also under sulphated. *Biochimie* **85**, 101–107 (2003)
16. Mizon, C., Mairie, C., Balduyck, M., Hachulla, E., Mizon, J.: The chondroitin sulfate chain of bikunin-containing proteins in the inter-alpha-inhibitor family increases in size in inflammatory diseases. *Eur. J. Biochem.* **268**, 2717–2724 (2001)
17. Kato, Y., Kudo, M., Shinkawa, T., Mochizuki, H., Isaji, M., Shiromizu, I., Hoshida, K.: Role of O-linked carbohydrate of human urinary trypsin inhibitor on its lysosomal membrane-stabilizing property. *Biochem. Biophys. Res. Commun.* **243**, 377–383 (1998)
18. Hochstrasser, K., Schoenberger, O.L., Rossmanith, I., Wachter, E.: Kunitz-type proteinase inhibitors derived by limited proteolysis of the inter-alpha-trypsin inhibitor. V. Attachments of carbohydrates in the human urinary trypsin inhibitor isolated by affinity chromatography. *Hoppe-Seyler Z. Physiol. Chem.* **362**, 1357–1362 (1981)
19. Suzuki, M., Kobayashi, H., Tanaka, Y., Hirashima, Y., Terao, T.: Structure and function analysis of urinary trypsin inhibitor (UTI): identification of binding domains and signaling property of UTI by analysis of truncated proteins. *Biochim. Biophys. Acta* **1547**, 26–36 (2001)
20. Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S., et al.: Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 16899–16903 (2002)
21. van Rooijen, J.J.M., Kamerling, J.P., Vliegenthart, J.F.G.: The abundance of additional N-acetyllactosamine units in N-linked tetraantennary oligosaccharides of human Tamm–Horsfall glycoprotein is a donor-specific feature. *Glycobiology* **8**, 1065–1075 (1998)
22. van Rooijen, J.J.M., Kamerling, J.P., Vliegenthart, J.F.G.: Sulfated di-, tri- and tetraantennary N-glycan in human Tamm–Horsfall glycoprotein. *Eur. J. Biochem.* **256**, 471–487 (1998)
23. van Rooijen, J.J.M., Voskamp, A.F., Kamerling, J.P., Vliegenthart, J.F.G.: Glycosylation sites and site-specific glycosylation in human Tamm–Horsfall glycoprotein. *Glycobiology* **9**, 21–30 (1999)
24. Huang, Z.Q., Sanders, P.W.: Localization of a single binding site for immunoglobulin light chains on human Tamm–Horsfall glycoprotein. *J. Clin. Invest.* **99**, 732–736 (1997)
25. Jovine, L., Qi, H., Williams, Z., Litscher, E., Wasserman, P.M.: The ZP domain is a conserved module for polymerization of extracellular proteins. *Nat. Cell. Biol.* **4**, 457–461 (2002)

26. Fournier, T., Medjoubi-N, N., Porquet, D.: Alpha-1-acid glycoprotein. *Biochim. Biophys. Acta* **1482**, 157–171 (2000)
27. De Graaf, T.W., van der Stelt, M.E., Anbergen, M.G., van Dijk, W.: Inflammation-induced expression of sialyl Lewis X-containing glycan structures on alpha-1-acid glycoprotein (orosomucoid) in human sera. *J. Exp. Med.* **177**, 657–666 (1993)
28. Cioci, G., Rivet, A., Koca, J., Serge, P.: Conformational analysis of complex oligosaccharides: the CICADA approach to the uromodulin O-glycans. *Carbohydr. Res.* **339**, 949–959 (2004)
29. Fukuoka, S.I., Kobayashi, K.I.: Analysis of the C-terminal structure of urinary Tamm–Horsfall protein reveals that the release of the glycosyl phosphatidylinositol-anchored counter part from the kidney occurs by phenylalanine-specific proteolysis. *Biochem. Biophys. Res. Commun.* **289**, 1044–1048 (2001)
30. Afonso, A.A.M., Charlwood, P.A., Marshall, R.D.: Isolation and characterization of glycopeptides digests for human Tamm–Horsfall glycoproteins. *Carbohydr. Res.* **89**, 309–319 (1981)
31. Azuma, Y., Murata, M., Matsumoto, K.: Alteration of sugar chains on alpha-1-acid glycoprotein secreted following cytokine stimulation of HuH-7 cells *in vitro*. *Clin. Chem. Acta* **294**, 93–103 (2000)
32. Nishi, K., Maruyama, T., Halsall, H.B., Handa, T., Otagiri, M.: Binding of alpha-1-acid glycoprotein to membrane results in a unique structural change and ligand release. *Biochemistry* **43**, 10513–10519 (2004)
33. Kopeck, V., Ettrich, R., Hofbauerova, K., Baumruk, V.: Structure of human alpha-1-acid glycoprotein and its high-affinity binding site. *Biochem. Biophys. Res. Commun.* **300**, 41–46 (2003)
34. Higai, K., Azuma, Y., Aoki, Y., Matsumoto, K.: Altered glycosylation of alpha-1-acid glycoprotein in patients with inflammation and diabetes mellitus. *Clin. Chim. Acta* **329**, 117–125 (2003)
35. Kunin, C.M.: Detection, Prevention, and Management of Urinary Tract Infection, 4th edn. pp. 39, 140–145, 316. Lea and Febiger, Philadelphia (1987)
36. Strauss, J.H., Strauss, E.G.: Viruses and Human Disease, 1st edn. Academic, San Diego (2002)
37. Cavallone, D., Malagolini, N., Monti, A., Wu, X.R., Serafini-Cessi, F.: Variation of high mannose chains to Tamm–Horsfall glycoprotein confers differential binding to type I-fimbriated *Escherichia coli*. *J. Biol. Chem.* **279**, 216–222 (2004)